



PCT/AU99/00424

AU99/424  
09/701818  
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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 3855 for a patent by GRADIPORE LIMITED filed on 2 June 1998.

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# AUSTRALIA

## Patents Act 1990

GRADIPORE LIMITED

### PROVISIONAL SPECIFICATION

*Invention Title:*

*Purification of antibodies*

The invention is described in the following statement:

### Technical Field

The present invention relates to methods suitable for purification of antibodies, particularly monoclonal antibodies from ascites fluid.

### Background Art

5       The processing of complex biological solutions is a major bottleneck in the biotechnology industry. Currently, there is a strong demand for cost effective technologies for the purification of naturally occurring and recombinant proteins (1, 2). This is particularly true for monoclonal antibodies, where there has been an on-going search for a simple generic  
10   method of purification. Much progress has been made in the last five years with many new methodologies, such as simulated moving-bed chromatography. Although these new processes have become increasingly effective in terms of yield and recovery, they often utilise harsh pH or ionic strength conditions for elution which may not always be compatible for  
15   maintaining maximal biological activity.

      The present inventors have found that Gradiflow technology (AU 601040) is particularly suitable for the purification of several different monoclonal antibodies. In contrast to conventional methods, Gradiflow uses mild, non-denaturing buffers. The present inventors have now found that the  
20   large size and characteristic pI of mouse antibodies make them an ideal target for the Gradiflow separation technology.

### Disclosure of Invention

      In a first aspect, the present invention consists in a method of separation of an antibody from a mixture of the antibody and at least one  
25   contaminant, the method comprising:

- (a) placing the antibody and contaminant mixture in a first solvent stream, the first solvent stream being separated from a second solvent stream by an electrophoretic membrane;
- (b) selecting the pH for the first solvent stream such that contaminants  
30   with pIs lower than the antibody will be charged;
- (c) applying an electric potential between the two solvent streams causing movement of at least some of the contaminants through the membrane into the second solvent stream while the antibody is substantially retained in the sample stream, or if entering the membrane, being substantially prevented  
35   from entering the second solvent stream;

(d) periodically stopping and reversing the electric potential to cause movement of any antibody having entered the membrane to move back into the first solvent stream, wherein substantially not causing any contaminants that have entered the second solvent stream to re-enter first solvent stream;

5 and

(e) repeating step (d) until the first solvent stream contains the desired purity of antibody.

Preferably, the mixture is a monoclonal antibody in ascitic fluid.

10 In a preferred embodiment of the first aspect, the electrophoretic membrane has a molecular mass cut-off of about 100 kDa. The pI of the antibody is usually obtained by isoelectric focusing (IEF). The pH of the first solvent stream is preferably about 8.0 to 8.5. Major protein contaminants including albumin whose pI is well known to be 5.5 were separated from the antibodies as they transferred into the second solvent stream at pH 8.3.

15 In a second aspect, the present invention consists in a method of separation of an antibody from a mixture of the antibody and at least one contaminant, the method comprising:

(a - e) separating the antibody according to the first aspect of the present invention;

20 (f) placing the separated antibody in a fresh first solvent stream, the first solvent stream being separated from a second solvent stream by an electrophoretic membrane;

(g) selecting the pH of the fresh first solvent stream such that the pH is lower than that used in step (b) but still greater than the pI of the antibody;

25 (h) applying an electric potential between the two solvent streams causing movement of at least some of the contaminants through the membrane into the second solvent stream while the antibody is substantially retained in the sample stream, or if entering the membrane, being substantially prevented from entering the second solvent stream;

30 (i) periodically stopping and reversing the electric potential to cause movement of any antibody having entered the membrane to move back into the first solvent stream, wherein substantially not causing any contaminants that have entered the second solvent stream to re-enter first solvent stream; and

35 (j) repeating step (i) until the first solvent stream contains the desired purity of antibody.

The molecular mass cut-off of the membrane used in step (f) is preferably larger than that used in step (b). A cartridge containing a large 1000 kDa pore size separating membrane has been found to be particularly suitable. The pH in step (g) is preferably from about 6 to 7.5. It will be appreciated that the pH of the buffer will depend on the pI of the antibody to be purified and those of the contaminants.

The present inventors have been able to obtain percent recoveries of monoclonal antibodies from ascitic fluid of at least 70% and greater than 90% using the methods according to the present invention.

In a third aspect, the present invention consists in use of Gradiflow in the purification and/or separation of antibodies.

In a fourth aspect, the present invention consists in an antibody purified by the method according to the first or second aspect of the present invention.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described in the following examples with reference to the accompanying drawings.

#### Brief Description of Drawings

Figure 1 shows the operating modes of the Gradiflow separating cartridge.

Figure 2 shows the IEF of the crude mouse ascites fluids to obtain the pIs of the specific antibodies of interest. Lanes 1 and 6 are markers, lanes 2 to 5 are starting materials separated by Gradiflow for antibodies labelled 1 to 4 in Table 1 respectively.

Figure 3 shows SDS-PAGE of the purified monoclonal antibodies. Lane 1 and 7 are the SDS-PAGE molecular weight markers. For contrast, the starting material for antibody 1 is placed in lane 2 while lanes 3, 4, 5 and 6 contain the final 4 product antibodies.

## Modes for Carrying Out the Invention

### GRADIFLOW TECHNOLOGY

The Gradiflow LM1000 is a preparative protein system that is simple to use and does not require harsh conditions for sample processing.

5       The membrane cartridges contain restriction and separating membranes which separate macromolecules on the basis of size or charge. The technology also has the capacity to simultaneously concentrate or desalt solutions containing macromolecules. The apparatus is controlled by a personal computer (as shown below in Figure 1) under a Windows 95 and  
10      Lab view format or can be manually configured with conventional pumps and power supply.

In contrast to conventional methods Gradiflow uses mild, non-denaturing buffers. The large size and characteristic pI of mouse antibodies make them an ideal target for the Gradiflow separation technology.

15       The Gradiflow is a preparative electrophoretic system. It can be used for protein separation and purification. Target proteins are separated on the basis of charge and/or size by using pore size separation membranes within an electric field. The separation cartridge is the core of the Gradiflow technology with each separation cartridge containing restriction and  
20      separating membranes. The role of the restriction membranes is to separate the sample and the product from the running buffer.

The Gradiflow can be operated in different modes. Figure 1 shows how the separation cartridge can be used to concentrate, desalt and to separate proteins or other macromolecules. These processes are possible due  
25      to the characteristics of the target proteins which differentiates them from other major protein contaminants, even in complex biological solutions. The characteristics such as size, charge, mobility as in terms of mass/charge ratios, shape and their pI can form the basis for the electrophoretic separation from other contaminants in the sample.

30       By selecting the pore size of the restriction membranes, proteins can remain in the sample stream while desalting occurs when smaller ions are removed by the running buffer stream (Figure 1a). Concentration of proteins by the Gradiflow is possible when the operator select the conditions such that proteins transfer across to a smaller volume in the product stream  
35      (Figure 1b). With the use of a separation membrane of chosen pore size (Figure 1c) in the cartridge, it is possible to concentrate as well as separate at

the same time. Similarly, the technology has the capacity to simultaneously concentrate and desalt samples.

### **Charge Based Separations**

For the purification of monoclonal antibodies, the present inventors  
5 have used the separation cartridge as in Figure 1d which demonstrates how a  
charge based separation will occur in the Gradiflow separation cartridge.  
The purification strategy which have been devised is based on the pI of the  
monoclonal antibody which was found by isoelectric focussing (IEF). The  
pH of the running buffer was adjusted so that contaminants with lower pI  
10 will move into the product stream while the antibodies were retained in the  
sample stream with a 100 kDa cut-off membrane. Major protein  
contaminants including albumin whose pI is well known to be 5.5 were  
separated from the antibodies as they transferred into the product stream at  
pH 8.3.

## **15 EXPERIMENTAL**

### **Apparatus**

The pI of the antibodies were found by running an IEF gel using Novex  
IEF apparatus and Novex anode and cathodic buffers. The running  
conditions for 2.5 hours comprised of one hour at 100V, 1 hour at 200V and  
20 500V for 30 minutes. The IEF gel was fixed with a solution of trichloroacetic  
acid with sulfo-salicylic acid before the staining with Gradipure<sup>TM</sup> Comassie  
Blue.

The proteins were then fractionated with the Gradiflow system  
LM1000. The Gradiflow LM1000 is a computer controlled with Windows 95  
25 in Lab View format. Alternatively, a low-cost manually configured  
separation cartridge is also available. The low cost unit will operate with a  
conventional pump and power supply.

### **Gradiflow Running Conditions**

The system was run at 250V with peltier cooling that maintained  
30 temperatures at 7 degrees for both sample and product streams.

Ascitic fluid samples were individually diluted with three volumes of a  
buffer containing 40 mM Tris Borate containing 1 mM EDTA pH8.3. The  
separation of each sample was carried out in this buffer for 30 to 40 min at  
200V using the Gradiflow separating membrane with a pore size of 100 kDa.  
35 Under these conditions, the albumin and other impurities rapidly migrated  
across the membrane leaving the purified antibody in the sample stream. For

higher purity, a second run was selected at a pH close to the pI of each specific antibody and 1000 kDa membrane was employed. In this case, 40 mM Tris buffer used was adjusted to the required pH with HCl. The run time was 40 min at 200V. The impurities migrate through the membrane and the antibody remains in the sample stream.

#### **Determining Antibody Purity and Recovery**

Gradipore 4-20%T SDS gels were used to determine the purification process occurring in the Gradiflow over a period of 40 minutes. The SDS-PAGE sample buffer from Gradipore was added 50 mM DTT before samples were boiled for 3 minutes and loaded onto the gels. The changes to sample purity at 10 minute intervals can be determined by comparing the amount of protein bands appearing downstream in time and the intensity of the antibody bands in the sample stream compared to the starting material.

Protein levels in the sample and product streams were monitored using a UV fixed wavelength spectrophotometer at 280 nm every ten minutes. A 1 mg/mL solution of mouse monoclonal antibodies was assumed to have an optical density (OD) of 1.2 absorbance units at 280 nm. A 486 IBM compatible PC computer was used to control the system and store data during each run.

At every ten minutes, 50 ul of the sample and the product stream were collected into eppendorf tubes, for subsequent determination of the purified monoclonal biological activity by ELISA testing.

#### **CHEMICALS AND REAGENTS**

##### **Antibodies**

The antibodies used in this study were all generated by conventional procedures and supplied to Gradipore by Agen Biomedical Ltd, Brisbane Australia as murine ascitic fluids. Table 1 contains the properties of the target monoclonal antibodies in each fluid.

##### **Biological Activity Assays by ELISA**

The antibody activity was determined by a two site EIA. Firstly Dako mouse immunoglobulins was made up using Gradipore PBS such that 10 mL of 1/1000 stock solution. The wells of 96 well microtitre ELISA plates were coated by pipetting 50 uL per well with Dako mouse immunoglobulins in PBS for 1 hour at room temperature (25°C). The PBS stock solution was made by dissolving one Gradipore PBS vial into 1 L of distilled water.



Tween PBS was made by adding 1 mL of Tween 20 (Sigma Chemical Corp, St Louis, Missouri) into 1 L of PBS. After an hour, excess antigen was removed by inverting and tapping the plate each of the three times the plate was washed with the 0.1% Tween PBS solution. Monoclonal antibodies were serially diluted with 0.1% Tween PBS and then detected by adding 50  $\mu$ L of each sample into each well before incubating for another hour at room temperature.

Unbound monoclonal antibodies were removed by washing with PBS Tween 3 times as described above before the plate was coated with 1/500 dilution of peroxidase conjugated (HRPO) rabbit anti-mouse immunoglobulin (DAKO) in PBS/Tween. The plates were then incubated for 20 minutes at room temperature. The plate was washed 3 times with PBS Tween and add 100  $\mu$ L of activated substrate solution to each well. The substrate was activated immediately before use by adding 20  $\mu$ L of a 3% solution of  $H_2O_2$  to 10 mL of the substrate containing 10 mM citrate, 2.5 mM O-tolidine dihydrochloride and 0.025 mM EDTA pH 4.5 (ABTS). The colour reaction was stopped after 10 minutes by the addition of 50  $\mu$ L of 3.9% Oxalic acid which caused a colour change from clear to green and absorbance was recorded at dual wavelengths of 405 nm and 650 nm on a BioRad gel plate reader system.

#### **Mass Spectrophotometry**

The samples in 80 mM Tris EDTA Borate obtained from the Gradiflow were exchanged with 10 mM Tris HCL pH 8.0 before the sample was put into the mass spectrophotometer for further analysis of sample purity.

## **RESULTS**

### **One step purification of mouse antibodies**

The first purification step was to remove most of these contaminants at a pH where most proteins will be charged. By using a 100 kDa pore size membrane, the target antibodies that have a higher molecular weight than the contaminant will remain in the sample stream while impurities will move across to the product stream.

Acceptable purity was achieved after 40 min. The time course provides an analysis of one of the four different monoclonal antibodies during the Gradiflow purification procedure. It indicates that for this antibody, purification was achieved even after 20 minutes although Gradiflow was allowed to run to completion until 40 minutes.

After this step, information about the pI of the antibodies will enable higher purity can be obtained by incorporating a second step that utilises a running buffer with a pH close to the pI of the target antibody.

**Two Step purification : optimising the purification of antibody number 1,2 3 and 4.**

Antibody No. 1 was single step, run at pH 8.3 with 100 kDa membrane.

Antibody No. 2 and No. 4 were purified using two steps, with a second step at pH 7.1.

Antibody No. 3 was purified using two steps, with the second step at pH 6.4. The SDS PAGE showed increase purity of the antibodies after another charged separation at their pI that has effectively removed any trace impurities that may have remained in the samples stream with the antibodies.

With this step, the present inventors have demonstrated that charge-based separation can be successfully applied to separate proteins. Native gels (N-PAGE) showed that antibodies under native conditions have molecular weights ranging from 250 to 160 kDa and by using a cartridge containing a large 1000 kDa pore size separating membrane, they are not limited in terms of pore size to transfer into the product stream. The antibodies did not move across the separating membrane because they are uncharged at a pH close that is to their pI.

The four purified antibodies from the second step (Figure 3) show that the target proteins have been purified from the remaining contaminants that may still be present after the first fractionation step.

Preliminary scale up results from 1 mL to 10 mL of mouse ascites fluid showed that there may be trace contaminants remaining after step 2 if larger quantities were processed at the same time of 40 minutes. It could be that a longer processing time is required for larger quantities of proteins. According to the peptide mass fingerprinting results obtained, these consisted of trace mouse serum albumin and glucosamine-fructose-6-phosphate aminotransferase (isomerising hexosephosphate aminotransferase), a common renal mouse protein with a pI of 6.32 and a molecular mass of 765 kDa. These initial results suggest that an additional step may be required to separate the target antibody from this contaminant.

**Recovery**

The recovery of the monoclonal antibodies were determined by comparing the final biological activity of the product from the Gradiflow with the starting material that was placed in the sample stream. Table 1 summarises the results obtained.

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Table 1. Recovery as determined by ELISA

Antibody	Isotype	pI*	RECOVERY	
			%	mg/mL ascitic fluid
D46.8.4D4/23	Ig G1	pH 7.3-7.5	94	8.9
D34.30.4C3/17	Ig G2a	pH 6.8-7.5	73	8.1
C39.19.3C2/40	Ig G2b	pH 6.4-6.6	79	10.8
J37.KHL.2D1/96	Ig G1	pH 6.6-7.0	71	10.0

\* Determined by IEF electrophoresis.

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## DISCUSSION

Gradiflow technology can be used to purify monoclonal antibodies from ascitic fluid with high percent recovery of target protein and activity. The Gradiflow has potential advantages in cost, speed and recovery over conventional purification methods. In addition the disposable nature of the cartridges eliminated the possibility of cross contamination between different antibodies and eliminates the need for expensive cleaning and maintenance procedures.

The technology utilises the advantages provided by molecular mass exclusion membranes with the power of electrokinetic separation. Target proteins at a preparative scale can be obtained as shown in this application with monoclonal antibodies.

Scale-up of the Gradiflow purification process is simple, by increasing the size of the membranes that comprise the separation cartridge. The present inventors have demonstrated that high purity and recovery can be achieved by a fast and effective two-step purification of monoclonal antibodies from complex murine ascites. Unlike affinity chromatography and ion exchange chromatography, there is no factors influencing protein interactions and adsorption. It is not necessary to add other substances to

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enable purification that requires additional complex downstream processing that will result in the loss of the target proteins or the biological activity.

5 The extent of therapeutic and diagnostic applications of monoclonal antibodies have been significant and far reaching ever since they have been discovered. Yet the purification of these molecules have remained a challenge due to their complexity. The Gradiflow application the present inventors have developed is an attractive alternative to conventional monoclonal antibody purification techniques. It may be possible to extend the application to purify sufficient quantities of other biologically significant  
10 proteins from more abundant contaminants in solution that could previously have masked their discovery. If major contaminants can be easily removed with little loss to the target protein, higher yields and recovery will be possible.

15 The Gradiflow separation strategy can be expanded to a wide range of other samples with little sample treatments required before and after fractionation. This application demonstrates that the Gradiflow can be considered a cost effective alternative to protein A chromatography.

20 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this second day of June 1998

GRADIPORE LIMITED

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